Cytotoxic and Antimalarial β-Carboline Alkaloids from the Roots of *Eurycoma longifolia*

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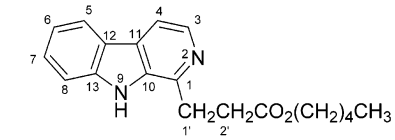
Received June 16, 2003

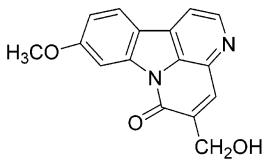
Three new [*n*-pentyl β-carboline-1-propionate (**1**), 5-hydroxymethyl-9-methoxycanthin-6-one (**2**), and 1-hydroxy-9-methoxycanthin-6-one (**3**)] and 19 known β-carboline alkaloids were isolated from the roots of *Eurycoma longifolia*. The new structures were determined by comprehensive analyses of their 1D and 2D NMR and mass spectral data and by chemical transformation. These compounds were screened for *in vitro* cytotoxic and antimalarial activities, and 9-methoxycanthin-6-one (**4**) and canthin-6-one (**5**) demonstrated significant cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines.

Eurycoma longifolia Jack (Simaroubaceae), also identified by the local names Tongkat Ali (in Malaysia) and Pasakbumi (in Indonesia), is a tall, slender shrub-tree, commonly found along the hilly jungle slopes of Malaysia and also widely distributed in primary and secondary, evergreen, and mixed deciduous forests in Burma, Indochina, Thailand, Sumatra, Borneo, and the Philippines. The roots are used as traditional treatments for dysentery, glandular swelling, persistent fever, and tertian malaria.[1−3] In addition, this plant is reputed to increase male virility and sexual prowess and has gained notoriety as a male aphrodisiac.[4−7] Various compounds isolated from this plant have shown antimalarial, cytotoxic, antiulcer, and antipyretic properties.[8−11]

In a continuing collaborative search for naturally occurring medicinal agents, the roots of Malaysian *E. longifolia* were investigated. Previous studies led to the isolation of several quassinoids and canthin-6-one alkaloids.[12−17] Recently, the cytotoxicity of several canthin-6-one alkaloids isolated from this plant was reported using a panel of cell lines comprising a number of human cancer cell types.[12] We now report the isolation and identification of three new β-carbolines from *E. longifolia*, together with cytotoxic, anti-HIV, and antimalarial evaluation of selected alkaloids from this plant.



1



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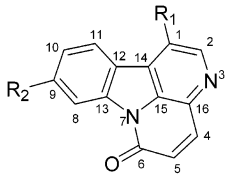

3: R_1 = OH, R_2 = OCH_3
4: R_1 = H, R_2 = OCH_3
5: R_1 = R_2 = H

Results and Discussion

Dried and powdered roots of *E. longifolia* were extracted with methanol under reflux and partitioned successively with chloroform and *n*-butanol. Repeated chromatography of the chloroform fraction provided three new (**1**−**3**) and 15 known β-carbolines, and purification of the *n*-butanol extract gave four previously reported alkaloids.

Compound **1** was obtained as a yellow powder. The pseudomolecular ion peak at *m*/*z* 311.1759 in its HR-FABMS corresponded with the molecular formula $C_{19}H_{22}N_2O_2$. The UV spectrum of **1** exhibited characteristic absorptions of a β-carboline chromophore at 247, 289, 303, and 376 nm.[18] IR absorption bands at 3346 and 1730 cm^{-1} indicated the presence of amino and ester carbonyl functionalities, respectively. In the ^1H NMR spectrum, a set of four mutually coupled symmetrical AA′, BB′ type aromatic protons at δ 8.21 (1H, d, *J* = 7.4 Hz), 7.26 (1H, dd, *J* = 7.4, 7.0 Hz), 7.54 (1H, dd, *J* = 8.3, 7.0 Hz), and 7.63 (1H, d, *J* = 8.3 Hz), which were assignable to H-5, H-6, H-7, and H-8, respectively, were indicative of the unsubstituted aromatic ring of a β-carboline. Two *ortho*-coupled doublets at δ 7.97 and 8.29 with a small coupling constant (*J* = 5.3 Hz) were characteristic of heteroaromatic protons, H-3 and H-4. Two downfield methylene triplets at δ 3.45 (2H, *J* = 7.3 Hz, H-1′) and 2.98 (2H, *J* = 7.3 Hz, H-2′) correlated with each other in the COSY spectrum and, together with a set of signals at δ 4.02 (2H, t, *J* = 6.5 Hz, H-4′), 1.53 (2H, m, H-5′), 1.35−1.22 (4H, m, H-6′, H-7′), and 0.84 (3H, t, *J* = 7.4 Hz) and a carbonyl carbon at δ 174.4, indicated the existence of a *n*-pentyl propionate side chain. This group was located on C-1 from the HMBC correlation between H-2′ and C-1. The connectivity between *n*-pentyl and propionate subunits was confirmed by the ^2J, ^3J-correlations of H-2′ and H-4′ with C-3′ in the HMBC spectrum. Thus, the structure of **1** was established as *n*-pentyl β-carboline-1-propionate and confirmed by esteri-

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10.1021/np030277n CCC: $25.00
Published on Web 09/25/2003

fication of the known β-carboline-1-propionic acid with *n*-pentanol. Spectral and physical data of isolated **1** coincided well with those of the semisynthetic sample.

Compound **2** was also isolated as a yellow powder. Its molecular formula $C_{16}H_{12}N_2O_3$ was determined on the basis of HRFABMS data. The UV spectrum of **2** displayed absorption maxima at 263, 273, 299, 309, 346, and 376 nm, which were similar to those reported for canthin-6-one alkaloids.[19] An IR absorption band at 1664 cm^{-1} revealed the presence of an amide carbonyl group, which was further supported by a carbon signal at δ 158.4 in the ^{13}C NMR spectrum. In the ^1H NMR spectrum, a set of ABX protons at δ 8.29 (1H, d, J = 8.7 Hz, H-11), 8.09 (1H, d, J = 2.3 Hz, H-8), and 7.20 (1H, dd, J = 8.7, 2.3 Hz, H-10) indicated the presence of a monosubstituted aromatic ring in **2**. HMBC correlation of a methoxy signal at δ 3.93 (s) with the carbon signal at δ 161.7 (C-9) confirmed that the aromatic ring was substituted with a methoxy group at C-9. Vicinal doublets at δ 8.78 (1H, J = 5.0 Hz, H-1) and 8.17 (1H, J = 5.0 Hz, H-2) were typical of heteroaromatic protons of canthin-6-ones. A broad singlet at δ 8.02 (H-4), a broad doublet at δ 4.60 (2H, J = 5.4 Hz, 5-CH_2OH), and a D_2O exchangeable triplet at δ 5.55 (1H, J = 5.4 Hz, 5-CH_2O*H*) suggested a hydroxymethyl-substituted lactam ring. The placement of the hydroxymethyl group on C-5 was confirmed by ^3J correlation of 5-CH_2OH with C-6 in the HMBC spectrum and a long-range COSY correlation between 5-CH_2OH and H-4. The remaining quaternary carbons, including C-12, -13, -14, -15, and -16, were assigned on the basis of HMQC and HMBC spectral data. Thus, the structure of **2** was elucidated as 5-hydroxymethyl-9-methoxycanthin-6-one.

Compound **3**, obtained as a yellow powder, mp 235−237 °C, was assigned the molecular formula $C_{15}H_{10}N_2O_3$ due to the presence of a pseudomolecular ion peak at *m*/*z* 267.0767 from its HRFABMS analysis. Its UV absorption maxima at 274, 283, 358, and 374 nm were similar to those reported for canthin-6-ones.[19] An IR absorption band at 1671 cm^{-1} combined with a carbon signal at δ 160.0 (C-6) indicated the presence of an amide carbonyl group. The ^1H NMR spectrum of **3** also exhibited a set of ABX protons at δ 8.10 (1H, d, J = 2.3 Hz, H-8), 8.09 (1H, d, J = 8.6 Hz, H-11), and 7.20 (1H, dd, J = 8.6, 2.3 Hz, H-10) and a methoxy signal at δ 3.92 (3H, s), which suggested that, like **2**, compound **3** possessed a 9-methoxycanthin-6-one skeleton. Isolated vicinal doublets at δ 8.04 (1H, J = 9.6 Hz, H-4) and 6.74 (1H, J = 9.6 Hz, H-5) were characteristic of *cis*-coupled protons on the conjugated lactam ring of a canthin-6-one. A singlet at δ 8.38 was assignable to H-2 and displayed a ^2J HMBC correlation with an oxygen-bearing carbon at δ 150.8 (C-1), which inferred that C-1 was hydroxylated. This assumption was also supported by the 0.2 ppm downfield shift of H-11. All connectivities were unambiguously assigned from HMQC, HMBC, $^1H-^1H$ COSY, and NOESY experimental data. The assignment of quaternary carbons was furnished from HMQC and HMBC spectral data. Thus, the structure of **3** was established as 1-hydroxy-9-methoxycanthin-6-one.

9-Methoxycanthin-6-one (**4**),[20] canthin-6-one (**5**),[16] methyl β-carboline-1-carboxylate,[18] 4, 5-dimethoxycanthin-6-one,[20] 10-methoxycanthin-6-one,[21] 8-hydroxy-9-methoxycanthin-6-one,[20] 9,10-dimethoxycanthin-6-one,[16] 5-methoxycanthin-6-one,[22] 9-hydroxycanthin-6-one,[20] 5-hydroxymethylcanthin-6-one,[23] canthin-6-one 3*N*-oxide,[16] 9-methoxycanthin-6-one 3*N*-oxide,[20] picrasidine Q,[24] β-carboline-1-propionic acid,[12] 7-methoxy-β-carboline-1-propionic acid,[12] canthin-6-one 9-*O*-β-glucopyranoside,[25] 9-hydroxycanthin-6-one 3*N*-oxide,[12] picrasidine L,[16] and 1-hydroxycanthin-6-one[26] were also isolated from the roots of *E. longifolia*. These known compounds were identified by comparison of their physical and spectral data with published values.

Table 1. Cytotoxicity of β-Carboline Alkaloids against Human Cancer Cell Lines A-549 and MCF-7

compound	ED_{50} in μg/mL (percentage of inhibition observed)	
	A-549	MCF-7
4	<2.5 (55)	4.5
5	3.6	7.3
9-hydroxycanthin-6-one	10.0	19.6
9-methoxycanthin-6-one 3*N*-oxide	18.5	18.9
picrasidine Q	16.2	18.1
β-carboline-1-propionic acid	NA	>20 (8)
7-methoxy-β-carboline-1-propionic acid	>20 (7)	>20 (15)
canthin-6-one 9-*O*-β-glucopyranoside	4.2	16.1

Furthermore, several of the isolated compounds were assayed for biological activity against different targets. Compounds **4**, **5**, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one 3*N*-oxide, picrasidine Q, β-carboline-1-propionic acid, 7-methoxy-β-carboline-1-propionic acid, and canthin-6-one 9-*O*-β-glucopyranoside were screened for *in vitro* cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines[27] and inhibition of HIV replication in H9 lymphocytes,[28] and compounds **1**, **4**, **5**, methyl β-carboline-1-carboxylate, 9-hydroxycanthin-6-one, 9-methoxycanthin-6-one 3*N*-oxide, picrasidine Q, β-carboline-1-propionic acid, 7-methoxy-β-carboline-1-propionic acid, and 9-hydroxycanthin-6-one 3*N*-oxide were evaluated for antimalarial activity against three *Plasmodium falciparum* clones, W2, D6, and TM91C235.[29] β-Carbolines **4** and **5** and canthin-6-one 9-*O*-β-glucopyranoside demonstrated strong cytotoxicity toward A-549 cell lines and weak cytoxicity toward MCF-7 cell lines (Table 1). None of the tested compounds showed significant anti-HIV effects. Compounds **5** and 9-hydroxycanthin-6-one exhibited very weak antimalarial activity against the W2 *P. falciparum* clone with IC_{50} values 2238 and 2336 ng/mL, respectively, and picrasidine Q against both the W2 (IC_{50} 3525 ng/mL) and D6 (IC_{50} 2957 ng/mL) clones.

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. UV spectra were recorded on a Hitachi UV-3210 spectrophotometer in MeOH. IR spectra were determined on a Shimadzu FT-IR 8501 spectrophotometer as KBr disks. ^1H and ^{13}C NMR spectra were obtained on Bruker Avance-300 and AMX-400 NMR spectrometers, with tetramethylsilane (TMS) as internal standard. FAB and HRFABMS were measured on a JEOL JMS-700 mass spectrometer.

Plant Material. The roots of *E. longifolia* were collected in Malaysia in January 2001 and authenticated by one of the authors (J.-B.W.). A voucher specimen (TSWu 20010005) has been deposited at the Herbarium of National Cheng Kung University, Tainan, Taiwan.

Extraction and Separation. The dried roots (10.5 kg) were cut into small pieces, extracted with MeOH (20 L × 7) under reflux, and concentrated under reduced pressure to give dark brown syrup (500 g). The crude extract was partitioned successively with $CHCl_3$ and *n*-butanol. The chloroform layer was concentrated *in vacuo* to leave a brown syrup (50 g), which was chromatographed on silica gel using gradients of $CHCl_3$ and acetone to give 12 fractions. Fraction 2 was rechromatographed on silica gel using $CHCl_3$−MeOH (99:1) as eluent to obtain methyl β-carboline-1-carboxylate (2.0 mg) and 4, 5-dimethoxycanthin-6-one (1.2 mg). Fraction 4 was repeatedly

chromatographed on silica gel using an eluent of *n*-hexane and EtOAc (2:1) to give **4** (125.5 mg) and **5** (180.0 mg). Fraction 5 was rechromatographed on silica gel eluted with a gradient of *n*-hexane and EtOAc to obtain 10-methoxycanthin-6-one (1.0 mg), 8-hydroxy-9-methoxycanthin-6-one (2.5 mg), 9,10-dimethoxycanthin-6-one (2.0 mg), and 5-methoxycanthin-6-one (1.5 mg), successively. Silica gel column chromatography of fraction 6 and further purification with preparative thin-layer chromatography using a mixture of *n*-hexane and EtOAc (2:1) gave **1** (3.3 mg). Fraction 8 was also subjected to column chromatography over silica gel with diisopropyl ether−MeOH (9:1) to afford 9-hydroxycanthin-6-one (90.8 mg), 5-hydroxymethylcanthin-6-one (1.5 mg), and canthin-6-one 3*N*-oxide (1.2 mg). Fraction 9 was rechromatographed on a silica gel column eluting with *n*-hexane−EtOAc (2:1) to yield **2** (3.5 mg), **3** (3.8 mg), and 9-methoxycanthin-6-one 3*N*-oxide (10.2 mg). Fraction 11 was separated by column chromatography on reversed C-18 gel eluting with water and a step gradient of MeOH to afford picrasidine Q (1.0 mg), β-carboline-1-propionic acid (10.3 mg), and 7-methoxy-β-carboline-1-propionic acid (10.5 mg).

The butanol layer (230 g) was directly chromatographed on a Diaion HP-20 column eluting with a gradient of water and MeOH to give six fractions. Fractions 4 and 5 were investigated for β-carbolines because they reacted positively with Dragendorff reagent and displayed yellow or blue color when irradiated with UV light (360 nm). Silica gel column chromatography of fraction 4 with mixed eluents of $CHCl_3$ and MeOH (9:1) saturated with water yielded canthin-6-one 9-*O*-β-glucopyranoside (20.5 mg), 9-hydroxycanthin-6-one 3*N*-oxide (6.0 mg), and picrasidine L (1.2 mg). Fraction 5 was treated similarly to afford 1-hydroxycanthin-6-one (1.5 mg).

Preparation of 1. A mixture of β-carboline-1-propionic acid (2.95 mg, 12.3 μmol), concentrated sulfuric acid (1.0 mL), and *n*-pentanol (2.0 mL) was heated at 80 °C for 4 h. The resulting mixture was decanted into 10 mL of cold water, and the aqueous solution was extracted with EtOAc (15 mL × 10). The organic layers were combined, repeatedly washed with brine, and dried over anhydrous Na_2SO_4. The dried filtrate was concentrated *in vacuo*, and the residue was subjected to preparative thin-layer chromatography developed with a mixture of *n*-hexane and EtOAc (2:1) to afford **1** (3.30 mg, 10.2 μmol, 83%).

***n*-Pentyl β-carboline-1-propionate (1):** yellow powder (benzene), mp 127 °C (dec); UV (MeOH) λ_{max} (log ϵ) 235 (3.90), 247 (3.85), 289 (3.60), 303 (3.50), 335 (3.07), 350 (3.08), 376 (2.93) nm; IR (KBr) ν_{max} 3346, 2926, 1730, 1628, 1459 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz) δ10.85 (1H, br s, D_2O exchangeable, NH), 8.29 (1H, d, J = 5.3 Hz, H-4), 8.21 (1H, d, J = 7.4 Hz, H-5), 7.97 (1H, d, J = 5.3 Hz, H-3), 7.63 (1H, d, J = 8.3 Hz, H-8), 7.54 (1H, dd, J = 8.3, 7.0 Hz, H-7), 7.26 (1H, dd, J = 7.4, 7.0 Hz, H-6), 4.02 (2H, t, J = 6.5 Hz, H-4′), 3.45 (2H, t, J = 7.3 Hz, H-1′), 2.98 (2H, t, J = 7.3 Hz, H-2′), 1.53 (2H, m, H-5′), 1.22−1.35 (4H, m, H-6′ and H-7′), 0.84 (3H, t, J = 7.4 Hz, H-8′); ^{13}C NMR (acetone-d_6, 75 MHz) δ 173.3 (C-3′), 144.3 (C-1), 142.1 (C-13), 137.3 (C-3), 135.3 (C-10), 129.7 (C-11), 129.4 (C-7), 122.6 (C-5), 122.4 (C-12), 120.7 (C-6), 114.0 (C-8), 112.9 (C-4), 64.5 (C-4′), 32.4 (C-5′), 31.7 (C-2′), 31.4 (C-6′), 28.4 (C-1′), 19.7 (C-7′), 13.9 (C-8′); FABMS m/z 311 [M + H]$^+$ (37), 149 (33), 117 (100); HRFABMS m/z 311.1759 [M + H]$^+$ (calcd for $C_{19}H_{23}N_2O_2$, 311.1760).

5-Hydroxymethyl-9-methoxycanthin-6-one (2): yellow powder (MeOH), mp 235 °C (dec); UV (MeOH) λ_{max} (log ϵ) 263 (3.24), 273 (3.35), 299 (2.99), 309 (3.03), 346 (3.02), 376 (2.83) nm; IR (KBr) ν_{max} 3360, 2920, 1664, 1458 cm^{-1}; ^1H NMR (DMSO-d_6, 400 MHz) δ 8.78 (1H, d, J = 5.0 Hz, H-1), 8.29 (1H, d, J = 8.7 Hz, H-11), 8.17 (1H, d, J = 5.0 Hz, H-2), 8.09 (1H, d, J = 2.3 Hz, H-8), 8.02 (1H, br s, H-4), 7.20 (1H, dd, J = 8.7, 2.3 Hz, H-10), 5.55 (1H, t, J = 5.4 Hz, D_2O exchangeable, 5-CH_2OH), 4.60 (2H, br d, J = 5.4 Hz, 5-CH_2OH), 3.93 (3H, s, 9-OCH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 161.7 (C-9), 158.4 (C-6), 146.0 (C-1), 141.3 (C-5), 140.3 (C-13), 135.3 (C-15), 133.1 (C-4), 130.7 (C-16), 129.2 (C-14), 124.6 (C-11), 117.5 (C-12), 115.7 (C-2), 113.2 (C-10), 101.1 (C-8), 58.7 (5-CH_2OH), 55.8 (9-OCH_3); FABMS m/z 281 [M + H]$^+$ (33), 221 (42), 207 (39), 176 (81), 154 (91), 147 (100); HRFABMS m/z 281.0926 [M + H]$^+$ (calcd for $C_{16}H_{13}N_2O_2$, 281.0926).

1-Hydroxy-9-methoxycanthin-6-one (3): yellow powder (MeOH), mp 235−237 °C; UV (MeOH) λ_{max} (log ϵ) 274 (3.05), 283 (3.15), 358 (3.11), 374 (3.06) nm; IR (KBr) ν_{max} 2924, 1671, 1605, 1460 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.38 (1H, s, H-2), 8.10 (1H, d, J = 2.3 Hz, H-8), 8.09 (1H, d, J = 8.6 Hz, H-11), 8.04 (1H, d, J = 9.6 Hz, H-4), 7.17 (1H, dd, J = 8.6, 2.3 Hz, H-10), 6.74 (1H, d, J = 9.6 Hz, H-5), 3.92 (3H, s, 9-OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 160.8 (C-9), 160.0 (C-6), 150.8 (C-1), 139.9 (C-4), 139.5 (C-15), 135.9 (C-2), 128.2 (C-16), 125.0 (C-11), 123.5 (C-5), 116.9 (C-13), 114.8 (C-14), 113.6 (C-12), 113.5 (C-10), 101.3 (C-8), 56.1 (9-OCH_3); FABMS m/z 267 [M + H]$^+$ (21), 221 (39), 207 (46), 165 (50), 147 (100); HRFABMS m/z 267.0767 [M + H]$^+$ (calcd for $C_{15}H_{11}N_2O_3$, 267.0770).

Biological Assays. *In Vitro* Cytotoxicity Assay. Cytotoxic assays were performed as described in Rubinstein *et al.*[27] The cell lines used were human lung cancer (A549) and human breast cancer (MCF-7). Cytotoxicity, ED_{50} for each cell line, is the concentration of compound that causes a 50% reduction in adsorbance at 562 nm relative to untreated cells using the sulforhodamine B assay.

Anti-HIV Evaluation. Inhibition of HIV replication was evaluated in the H9 lymphocyte T cell line using a p24 antigen ELISA assay as previously described.[28] P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. AZT was also assayed during each experiment as a positive drug control.

Antimalarial Assay. *In vitro* antimalarial drug susceptibility was assayed as previously described[29] using a modified procedure first published by Desjardins *et al.*[30] with modifications developed by Milhous *et al.*[31] The assay is based on the incorporation of radiolabeled hypoxanthine by the parasite with known antimalarials mefloquine and choroquine assayed in parallel as positive controls.

Acknowledgment. The authors are grateful for financial support from the National Science Council, Taiwan (NSC 91-2113-M-006-007), awarded to T.-S.W. and for partial support from NIH grants CA17624 and AI33066 awarded to K.-H.L.

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NP030277N